Chapter 6

Serine proteases — Cloning, Expression and Potential Applications

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Additional information is available at the end of the chapter

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1. Introduction

1.1. Snake venom serine proteases

Serine proteases have been isolated from the venoms of viperidae snakes [1, 2] and affect several physiological processes such as the coagulation cascade. These enzymes are called snake venom serine proteases (SVSPs), they are multi-functional proteins with a catalytic triad formed by HDS amino acids [3].

The SVSPs resembles at least in part thrombin, a multifunctional protease that plays a key role in coagulation. Therefore these enzymes are denominated snake venom thrombin-like enzymes (SVTLEs), and are widely distributed in the venoms of several genera [4,5]. While thrombin is able to cleave both fibrinopeptide A (FPA) and fibrinopeptide B (FPB) from fibrinogen leading the formation of fibrin and activating factor XIII, some actions of SVTLEs usually cleave FPA alone and only a few cleave FPB. Thus, without cleavage of both FPA and FPB they are unable to activate factor XIII producing fibrin monomers that are not cross-linked, leading to clots markedly susceptible to digestion by plasmin and are rapidly removed from circulation by either reticuloendothelial phagocitosis and/or normal fibrinolysis. This process causes a breakdown in the fibrinolytic system and effective removal of fibrinogen from the plasma [6].

2. Body

There are three groups of snake venom fibrinogen clotting enzymes based on the rates of release of fibrinopeptides A and/or B from fibrinogen. In addition to SVLTEs, other SVSPs

groups are active in other parts of the coagulation cascade, such as kallikrein-like enzymes (KN); plasminogen activators (PA); protein C like enzyme and factor V activators. One group releases fibrinopeptide A preferentially (the venombin A group including ancrod from venom of the Malayan pit viper, *Calloselasma rhodostoma*); another group releases both fibrinopeptides A and B (the venombin AB group including halystase and calobin from *Agkistrodon halys blomhoffii* and *Agkistrodon caliginosus*, respectively) and the third group releases fibrinopeptide B preferentially (the venombin B group including v enzyme from venom of the southern copperhead, *Agkistrodon contortrix contortrix*) [5,7,8]. Figure 1 summarizes some snake toxins that affect the blood coagulation cascade, based on [6].

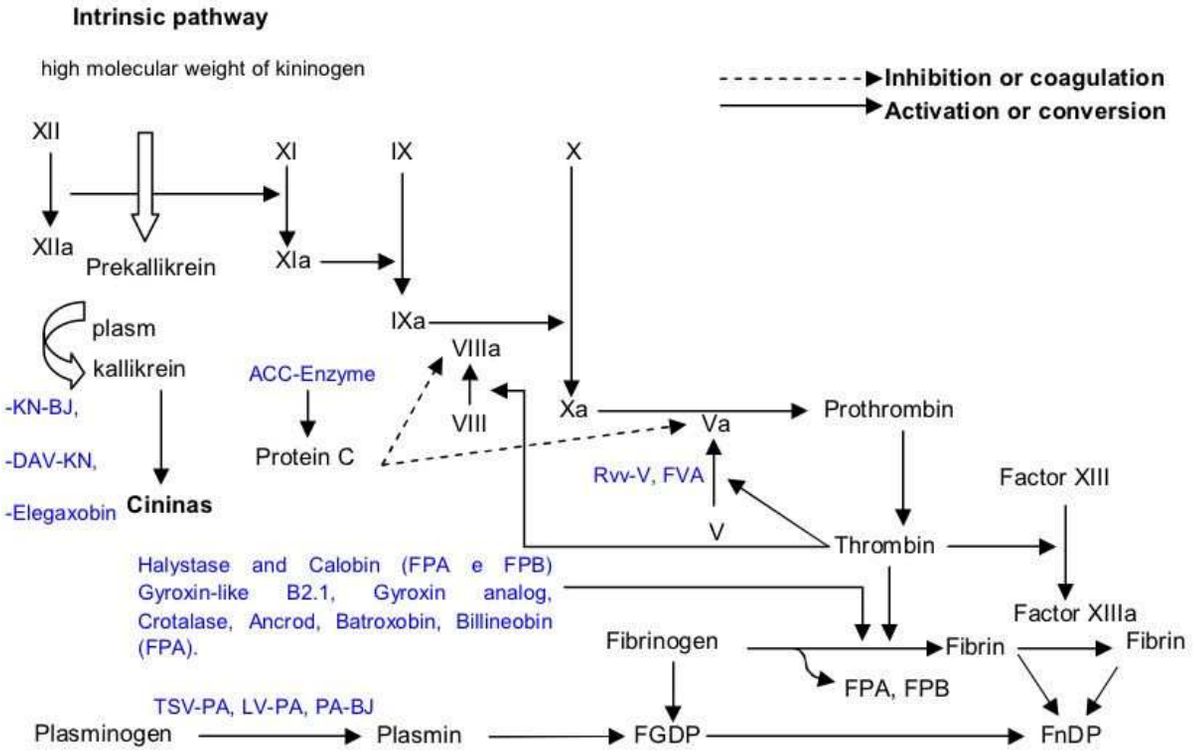

Figure 1. Some SVSPs acting in blood system. FGDP: Fibrinogen degradation products; FNDP: Fibrin degradation products; FPA: Fibrinopeptide A; FPB: Fibrinopeptide B. **KN-BJ**, *Bothrops jararaca* (O13069) [9]; **Dav-KN**, *Agkistrodon acutus* (Q9I8X0) [10]; **Elegaxobin-1**, *Trimeresurus elegans* (P84788) [11]; **ACC-C Protein C activator;** *Agkistrodon contortrix contortrix* (P09872) [12]; **RVV-Vα** Russel's viper venom FV activator alpha, *Daboia russelli siamensis* (P18964) [13]; **FVA Factor V-activating enzyme**, *Vipera lebetina* (Q9PT41) [14]; **Halystase**, *Agkistrodon halys blomhoffii* (P81176) [15]; **Calobin**, *Agkistrodon caliginosus* (Q91053) [16], **Gyroxin-like B2.1**, *Crotalus durissus terrificus* (Q58G94) [17]; **Gyroxin analog**, *Lachesis muta muta* (P33589) [18]; **Crotalase**, *Crotalus adamanteus* [19]; **Ancrod**, *Agkistrodon rhodostoma* (P26324) [20]; **Batroxobin**, *Bothrops atrox* (P04971) [21]; **Bilineobin**, *Agkistrodon bilineatus* (Q9PSN3) [22];**TSV-PA**, *Trimeresurus stejnegeri* (Q91516) [23]; **LV-PA**, *Lachesis muta muta* (P84036) [24]; **PA-BJ**, *Bothrops jararaca* (P81824) [25]. Toxin names were indicated in bold followed by snake species in italic and the Swissprot accession numbers were represented in parenthesis.

The major symptoms from snakebite affecting the haemostatic system are: (a) reduced coagulability of blood, resulting in an increased tendency to bleed, (b) bleeding due to the damage to blood vessels, (c) secondary effects of increased bleeding, ranging from hypovo-

laemic shock to secondary-organ damage, such as intracerebral haemorrhage, anterior pituitary haemorrhage or renal damage and (d) direct pathologic thrombosis and its sequelae, particularly pulmonary embolism [26].

The venom fibrinogenolytic serine proteases as well as the venom plasminogen activator, share extensive sequence homology with the thrombin-like venom serine proteases [27] such as ancrod [20, 28], batroxobin [21, 29], crotalase [30,31], gyroxin-like serine proteases [17], kallikrein-like enzyme from *Crotalus atrox* [32] and the protein C activator from *A. c. contortrix* [33] venoms.

The SVSPs share the conserved catalytic triad formed by the amino acids His, Asp and Ser, six disulfide bonds and global highly similarities, as seen in Figure 2. In this alignment, the deduced amino amino acid sequences of gyroxin-like B2.1, B1.3, B1.4, and B1.7 are highly similar to other SVSPs with several biological functions [17].

In order to predict the biological function of SVSPs, a functional dendrogram was generated based on the amino acid sequence alignment from Figure 2. Clearly there are subtle differences among these homologous enzymes that may explain different functions such as: fibrinogenolytic (group I), Aα fibrinogenases (subgroup I a), Protein C activator and CPI-enzyme (subgroup I b), kininogenases (subgroup I c), plasminogen activator (group II), factor V activators (group III) thrombin-like, or other specific enzymatic activities (Figure 3) [17].

Despite significant sequence identity (50–70%), SVSPs display high specificity toward distinct macromolecular substrates. Based on their biological roles, they have been classified as activators of the fibrinolytic system, procoagulant, anticoagulant and platelet-aggregating enzymes [34]. The procoagulant SVSPs activate FVII, FX and prothrombin [35] and shorten the coagulation times, while the anticoagulants inactivates factors Va and VIIIa and plays a key role in controlling haemostasis, Ancrod (from the Malayan pit viper, *Calloselasma rhodostoma*), in particular, has been used as an anticoagulant to achieve "therapeutic defibrination" [34].

As it can be seen in Figure 4 (top), the 3D model of gyroxin-like B2.1 shows the catalytic site (Ser184, His43 and Asp88) superimposed with the catalytic site of thrombin (Ser_{195}, His_{57} and Asp_{102}) [36]. The overall structure (bottom) show the typical fold of a serine proteinase in which the active-site cleft is located at the junction of the two six-stranded β-barrels. Among the conserved 3D structural features between trypsin-like enzyme and SVSPs are the two β-barrel subdomains, the orientation of catalytic site and the pattern of Cys residues. In contrast with other serine proteases, a unique long C-terminal tail of gyroxin are highly conserved only on SVSPs. In addition, SVSPs are active only as a single chain enzyme while prothrombin is activated by Factor Xa generating the Light (L) and Heavy (H) chains of active thrombin.

2.1. The role of Protease Activated Receptor (PAR) on serine protease coagulation

Protease-activated receptors (PARs) are members of family of seven-transmembrane G-protein-coupled receptors (GPCRs). The activation is triggered by the cleavage of the N-terminus of the receptor by a serine protease, resulting in the generation of a new tethered ligand that interacts with the receptor within its extracellular loop-2. This ligand binding to

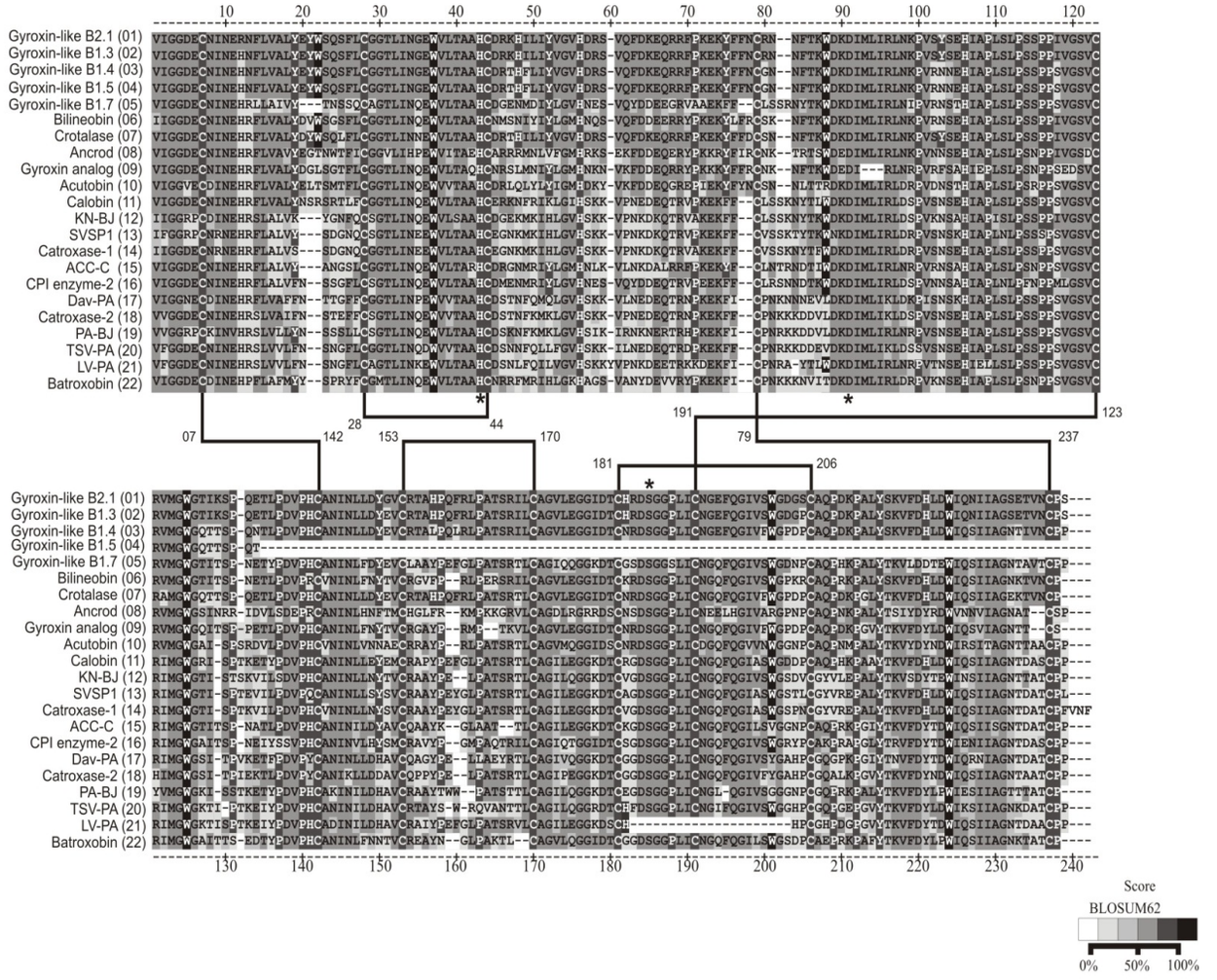

Figure 2. Alignment of snake venom serine proteases 1-5) Gyroxin-like B2.1, B1.3, B1.4, B1.5, B1.7 from *Crotalus durissus terrificus* (Q58G94, B0FXM1, B0FXM2, EU360953, B0FXM3, respectively). 6*)* Bilineobin from *Agkistrodon bilineatus* (Q9PSN3). 7) Crotalase from *Crotalus adamanteus*. 8) Ancrod from *Agkistrodon rhodostoma* (P26324). 9) Gyroxin analog from *Lachesis muta muta* (P33589). 10) Acutobin from *Agkistrodon acutus* (Q9I8X2). 11) Calobin from *Agkistrodon caliginosus* (Q91053). 12) KN-BJ from *Bothrops jararaca* (O13069). 13) SVSP-1 Venom serine proteinase from *Crotalus adamanteus* (Q8UUK2). 14) Catroxase-1 from *Crotalus atrox* (Q8QHK3). 15) ACC-C Protein C activator from *Agkistrodon contortrix contortrix* (P09872). 16) CPI enzyme from *Agkistrodon caliginosus* (O42207). 17) Dav-PA from *Agkistrodon acutus* (Q9I8X1). 18) Catroxase-2 from *Crotalus atrox* (Q8QHK2). 19) PA-BJ from *Bothrops jararaca* (P81824). 20) TSV-PA from *Trimeresurus stejnegeri* (Q91516). 21) LV-PA from *Lachesis muta muta* (P84036). 22) Batroxobin from *Bothrops atrox* (P04971). Indicated accession numbers are from Swissprot. The lines indicate the disulfide bonds and the catalytic triad (His, Asp and Ser) are represented by *. Toxin names were indicated in bold followed by snake species in italic and the Swissprot accession numbers were represented in parenthesis.

the core of PARs initiates an intracellular signal transduction pathway, which stimulates phosphoinositide breakdown and cytosolic calcium mobilization [37].

There are four PARs (PAR-1, PAR-2, PAR-3 and PAR-4), PAR-1, PAR-3 and PAR-4 can be activated by thrombin while PAR-2 is activated by trypsin and trypsin-like proteases, but not by thrombin [38]. PAR-1 is important for activation of human platelets by thrombin, but plays no apparent role in mouse platelet activation [39]. The consensus sequence among all the

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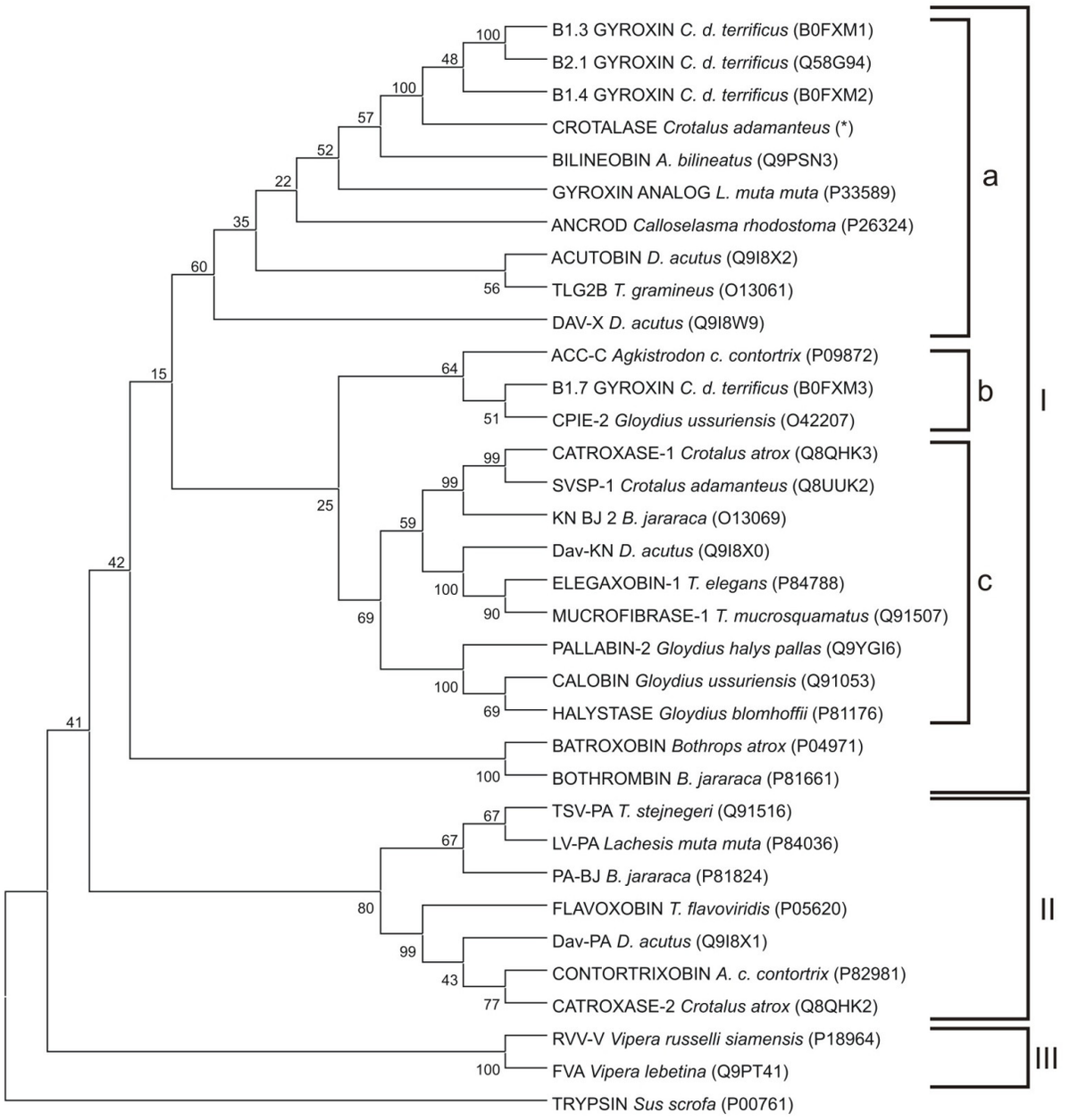

Figure 3. Dendrogram of 34 mature snake venom serine proteases. Toxin names were indicated in bold followed by snake species in italic and the Swissprot accession numbers were represented in parenthesis. (*) Crotalase toxin sequence was based on [19]. All positions containing gaps and missing data were eliminated from the dataset. There were a total of 188 positions in the final dataset. The distance was calculated by number of amino-acid differences. The optimal tree with the sum of branch length = 796.39 and the percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. Fibrinogenolytic (group I), Aα fibrinogenases (subgroup I a), Protein C activator and CPI-enzyme (subgroup I b), kininogenases (subgroup I c), plasminogen activator (group II), factor V activators (group III).

human PAR-1 activating peptides is XFXXR, indicating that the second residue (Phe) and fifth residue (Arg) are critical for the agonist binding and activation [40].

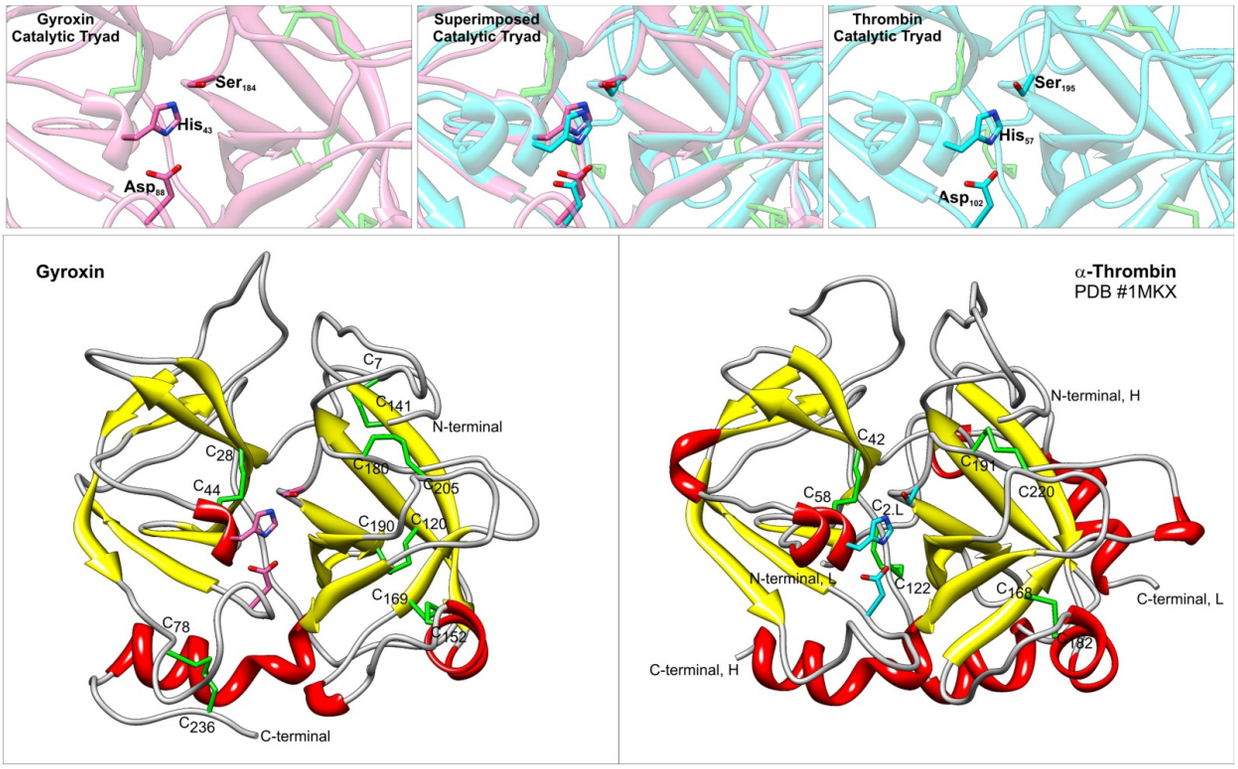

Figure 4. Gyroxin and bovine a-Thrombin (1mkx). The gyroxin homology model was based on crystallographic structures of *Trimeresurus stejnejeri* TSV-PA (1bqy), *Agkistrodon acutus* AaV-SP I (1op0) and *Agkistrodon acutus* AaV-SP II- DAV-PA (1op2). Top: Catalytic tryad superimposition of gyroxin residues (pink) and α-thrombin (blue). Bottom: Two β-barrel domains formed by β-sheets are depicted in yellow and α-helixes in red. Disulfid bridges are depicted in green. The residues from active site are showed in pink to gyroxin molecule and blue to activated thrombin.The C-teminus and N-terminus are indicated to both thrombin heavy (H) and ligth (L) chains and to gyroxin model.

It is known that the inhibitory effects of PAR1 antagonists on platelet aggregation caused by high concentrations of thrombin are limited but can be enhanced by combination with PAR4-blocking antibody, suggesting that simultaneous blockade of PAR1 and PAR4 may provide more effective antithrombotic therapy [41].

An example of snake venom serine protease that acts on coagulation through PAR is gyroxin (serine protease from *C.d.terrificus*) that promotes platelet aggregation through its involvement with PAR 1 and 4 [42]. In fact, a significant inhibition of the maximum platelet aggregation effect induced by gyroxin was observed in the presence of inhibitors of both PAR-1 [SCH79797] and PAR-4 [tcY-NH_2]. PAR-1 inhibitor was effective at concentration of about two orders of magnitude below than that required for PAR-4 inhibitor, and the combination of these two inhibitors were not capable to completely inhibit the platelet aggregation induced by gyroxin [42].

2.2. Molecular biology of SVSPs

Batroxobin (*Bothrops atrox* serine protease, E 3C.4.21.29) is a thrombin-like enzyme derived from *Bothrops atrox*, *moojeni* venom. In contrast to thrombin which converts fibrino-

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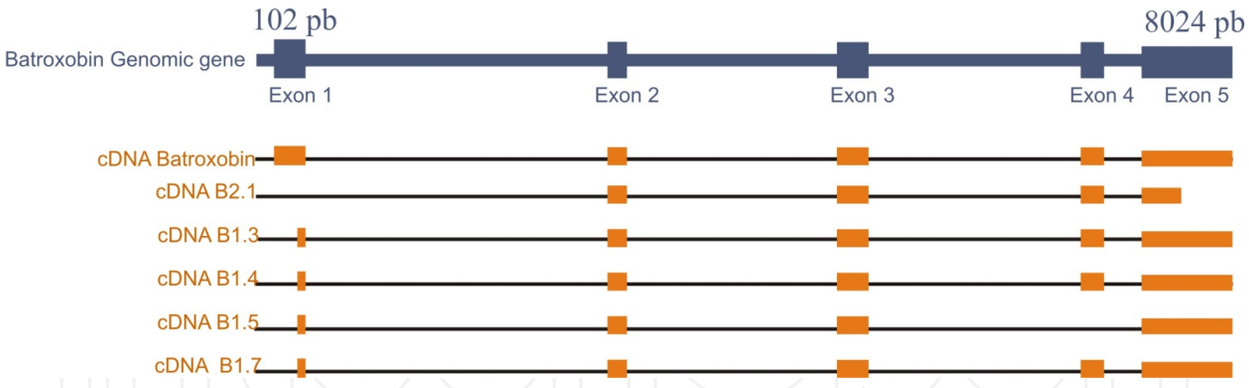

Figure 5. Organization of the batroxobin genomic gene, cDNA of Batroxobin and gyroxin-like B2.1, B.1.3, B1.4, B1.5 and B1.7 from *C.d.terrificus.*Blue boxes denote the location of exons of batroxobin genomic gene (X12747), the blue bars denote noncoding regions of introns. Orange boxes denote the location of exons of cDNA of Batroxobin (J02684) and gyroxin-like B1.3, B1.4, B1.5, B1.7 from *C.d.terrificus* (EU360951; EU360952; EU360953 and EU360954, respectively). In case of clone B2.1, only a partial sequence was obtained (GenBank accession number AY954040). The lack of exon 4 in B1.5 clone is because this clone is truncated by the insertion of a stop codon in translated sequence at the position 472 pb due to the joining of the exon 3 and exon 5.

gen into fibrin by splitting off fibrinopeptides A and B, batroxobin only splits off fibrinopeptide A [43].

Batroxobin gene spans 8 kilobase pairs and contains five exons and its mature form is encoded by exons 2 to 5. The catalytic residues of batroxobin, His-41, Asp-86, and Ser-178, are encoded by separate exons, exons 2, 3, and 5, respectively [44].

The exon/intron organization of the batroxobin gene is different from that of the prothrombin gene but very similar to those of the trypsin and kallikrein genes. These results indicate that batroxobin is not a member of the prothrombin family but one of the trypsin kallikrein family. The snake venom gland is assumed to originate from the submaxillary gland. Therefore, batroxobin is expected to be a member of the glandular kallikrein family [44].

cDNA libraries of snake venom glands have been constructed from various species and several clones encoding SVSPs have been isolated and sequenced. SVSPs are one chain proteins encoded by cDNAs containing an open reading frame (ORF) around 800 bp. The 5′UTRs (5 ′untranslated region) are usually short while the 3′UTRs (3′untranslated region) vary in length and may contain more than 1200 nucleotides [3].

Snake venom serine protease are synthesized as zymogens of ~256–257 amino acids with a putative signal peptide of 18 amino acids and a proposed activation peptide of six amino acid residues [3]. In the process of protein export, a central role is played by the signal sequence: an N-terminal segment that somehow initiates export whereupon it is cleaved from the zymogen. Three structurally dissimilar regions have been recognized so far: a positively charged N-terminal region, a central hydrophobic region and a more polar C-terminal region that seems to define the cleavage site [45].

The organization of batroxobin gene, batroxobin cDNA and gyroxin-like B2.1, B1.3, B1.5 and B1.7 [17, 36, 44] are shown in Figure 5.

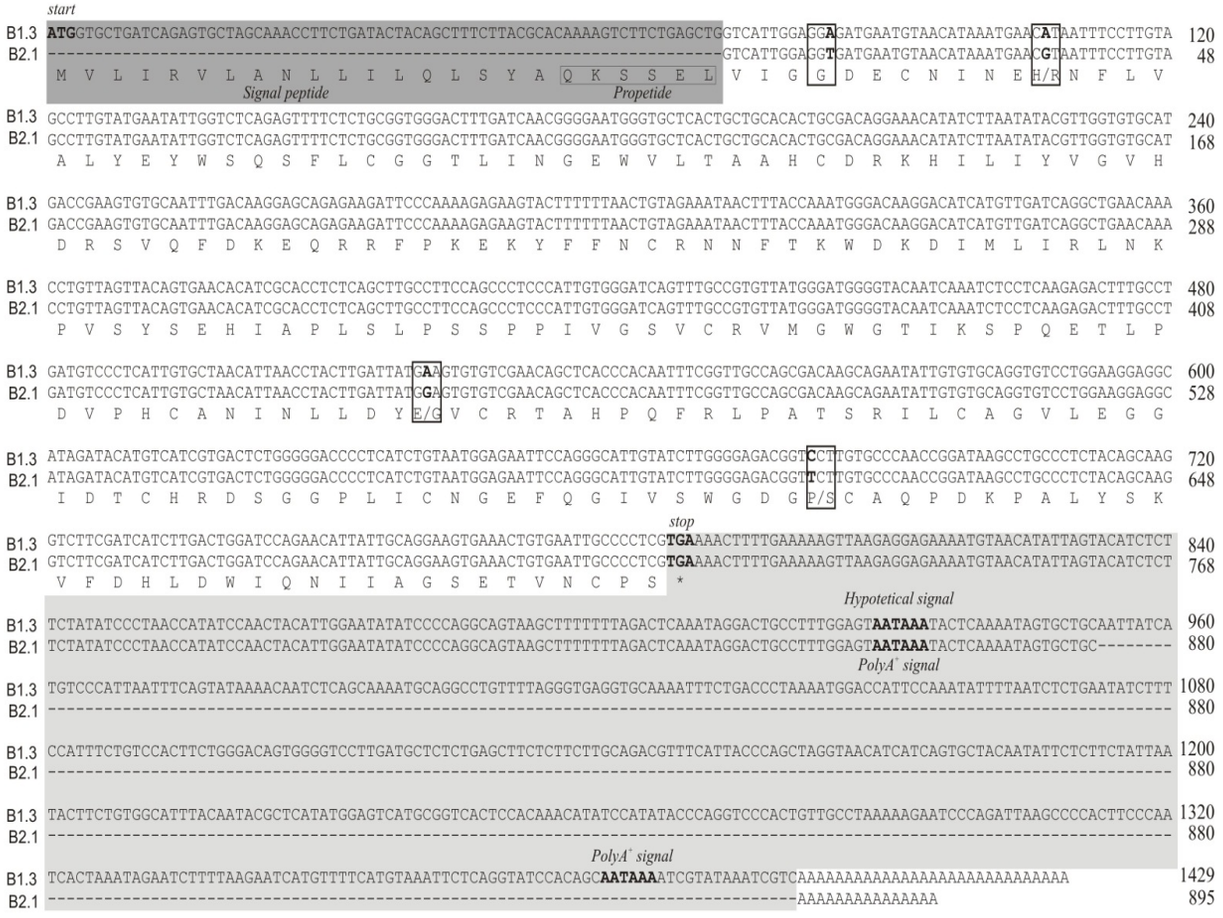

Figure 6. Alignment of nucleotide sequences from gyroxin-like B1.3 (EU360951) and B2.1 (AY954040). Coding region for signal peptide and propeptide is indicated in dark grey. Start codon and stop codons are in bold. The mature coding region is indicated in white. The mutations between B1.3 and B2.1 sequences are indicated by a box line and differences in nucleotides are in bold. Light grey encompasses the 3′ UTR. B1.3 hypothetical poly A+ signal (929–934 bp), B1.3 poly A^+ signal (1380–1385 bp) and B2.1 poly A^+ signal (857–862 bp) are in bold. Dashes represent gaps introduced for optimal sequence alignment.

Gyroxin-like B2.1 has a shorter 3′UTR compared with other clones. The lack of exon 4 in gyroxin-like B1.5 is because this clone is truncated by the insertion of a stop codon in translated sequence at the position 472 pb due to the joining of the exon 3 and exon 5 [17].

In Figure 6, the alignment of nucleotide sequences from gyroxin-like B1.3 and B2.1 revealed that clone B1.3 contains two consensus motifs for hypothetical poly(A^+) signals (5′- AATAAA -3′) at positions 929 and 1380 bp, whereas the B2.1 sequence contains only the first poly(A^+) signal at the position 857 bp and has a shorter 3′UTR and poly(A^+) tail [17].

The transcription of mRNA can be related with polyadenilation sites on 3′UTR (3′untranslated region). The presence of short and long 3′UTRs was also described for myogenin, Xmyog U_1 and Xmyog U_2 from *Xenopus laevis* (Xmyog U_2) [46] that contains one and two consensus motifs for a poly(A^+) signal, respectively. These results suggest the presence of at least, two different poly(A^+) signals in Xmyog U_2, generating two transcripts with different 3′ ends.

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Similarly, the presence of two signals of polyadenylation in gyroxin-like B1.3, suggests that two mRNAs could be transcripted with longer or smaller 3' UTR. Gyroxin-like B2.1 has only one signal of polyadenilation, showing a shorter 3´UTR than gyroxin-like B1.3 (Figure 5) [17].

2.3. Recombinant serine protease expression

Due to the great biotechnological potential of toxins present in the snakes venoms, many efforts have been made in order to clone and express those toxins in order to study its biological activity. However, the study of their properties is often hampered due to the small amount obtained and the difficulty of getting the animals to extract the poison, and when these are not the case, many toxins require several purification steps that result in a lower final yield. For these reasons, many toxin genes have been isolated, cloned and expressed in heterologous systems. This methodology not only make possible to obtain a large amount of toxins, but also enable amino acids modification by specific mutations in their DNA sequence. Thus, whole molecules may be broken down in order to study the function of its domains [47, 48], as well as amino acid residues may be exchanged for to study its role in substrate binding [49, 50].

2.3.1. Expression of serine protease on prokaryotic cells

Currently, the most used system to express snake toxins has been bacteria. However, the expression of recombinant SVSPs using *E. coli* as a host may result in expression of insoluble proteins that must be refolded *in vitro* in order to be activated. Batroxobin, for example, was the first SVSP to be expressed in insoluble form in *E. coli* and subsequently refolded to yield an active enzyme [51]. The plasminogen activator from *T. stejnegeri* was expressed in *E. coli*, but had to undergo a a denaturation-renaturation process in appropriate redox conditions to allow for the correct formation of disulfide bridges. Using an innovative method, a kallikrein-like protease (Tm-5) from the snake venom Taiwan habu (*Trimeresurus mucrosquamatus*), was expressed in *E.coli* by placing a polyhistidine-tag linked to an autocatalyzed site based on the cleavage specificity of the serine protease. The autocatalytic cleavage of Tm-5 from the polyhistidine-tagged fusion protein resulted in an active recombinant enzyme [53].

Acutin and mucrosobin, enzymes with fibrinogen-clotting and β-fibrinogenase activities respectively, were successfully expressed in *E. coli* [54,55]. The expression of a SVSP in *E.coli*, rCC-PPP, an isoform of cerastocytin from *Cerastes cerastes* with platelet-aggregating activity was reported [56]. After refolding, the recombinant enzyme showed to be a potent platelet proactivator and to clot fibrinogen.

2.3.2. Expression of serine protease on eukaryotic cells

In general SVSPs are glycosylated and this post-translational modification is important to the toxin activity, besides that, when expressed in *E. coli* those toxins frequently results in insoluble or inactive forms. Therefore the eukaryotic system such as yeast, mammalian cells and baculovirus expression system in insect cells have been explored, and although the number of works using this systems are small, they are growing substantially, mainly because of its

superior refolding machinery and post-translational modifications (e.g. phosphorylation and glycosylation) [57].

The recombinant Haly-PA was successfully expressed using the baculovírus expression system, displayed an indirect fibrinogenolytic activity depending on the presence of plasminogen and cleaved the plasminogen to generate the active plasmin. These results indicate that Haly-PA is a plasminogen activator and displays fibrinogenolytic activity through conversion of plasminogen to plasmin [58].

The recombinant Batroxobin from *Bothrops atrox moojeni* venom –expressed in *Pichia pastoris*, was able to coagulate plasma in a dose dependent manner. However, its molecular weight was higher than the native protein, indicating yeast-type carbohydrate in its structure [59].

The expression of a glycoprotein Gyroxin-like B2.1 from *Crotalus durissus terrificus* venom was reported in COS-7. In order to promote the secretion of this toxin to the culture medium it was fused to the IgK-chain secretion signal peptide at the N terminus [17]. The recombinant Gyroxin expressed in COS-7 cell (Figure 7-Western blot, lane 1) showed the same electrophoretic pattern of the native Gyroxin purified from the venom (Figure 7-Western blot, lane 2). Recombinant Gyroxin-like B2.1 was successfully achieved with esterase activity in the conditioned culture medium, as revealed by immunoblot of secreted protein and standard anti-crotalic serum from Butantan Institute (Figure 7).

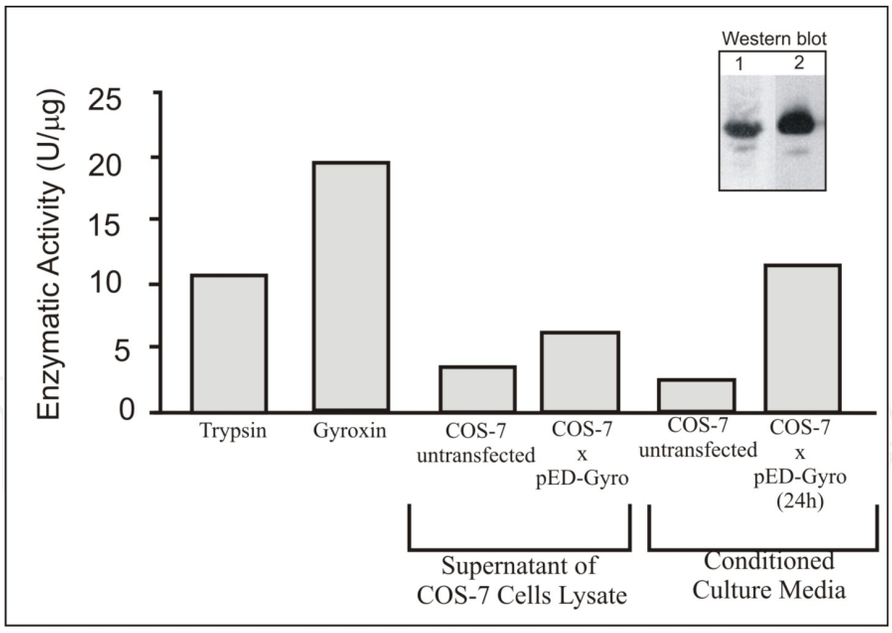

Figure 7. Esterase activity assay of recombinant Gyroxin purified by Benzamidine Sepharose from the supernatant of lysate and conditioned culture medium over 24 h of transfected COS-7 cells with pED-Gyro. Supernatant lysate and conditioned culture medium of untransfected COS-7 cells were used as negative controls. Porcine pancreas Trypsin and purified Gyroxin from *C.d.terrificus* venom were used as positive controls. 1- Western blot of COS-7 cells extract transfected with pED-Gyro., 2- 0.05 mg of Gyroxin purified from *C.d.terrificus* (positive control). The primary antibody was anti-crotalic serum from Butantan Institute and the reaction was detected with secondary antibody conjugated to horseradish peroxidase.

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Drug/trade name®	Target and function/treatment	Source
Ancrod (Viprinex)	Fibrinogen inhibitor/stroke	*Agkistrodon rhodostoma* (Malayan pit viper)
Batroxobin (Defibrase)	thrombin and protrhombin inhibitor/acute cerebral infarction, unspecific angina pectoris	*Bothrops moojeni*
Hemocoagulase	thrombin-like effect and thromboplastin activity/ prevention and treatment of haemorrhage	*Bothrops atrox*
Protac/protein C activator	protein C activator/clinical diagnosis of haemostatic disorder	*Agkistrodon contortix contortix* (American copperhead)
Reptilase	diagnosis of blood coagulation disorder	*Bothrops jararaca* (South American lance adder)
RVV-V	Proteolytic activation of factor V	*Daboia ruselli*

Table 1. Clinical applications and diagnostic kits from snake venom serine proteases.

2.4. Therapeutic and diagnostic use

Due to the properties of SVTLEs, they have been extensively investigated over the last decade for potential therapeutic and diagnostic use and some of them are summarized in Table 1, based on [6, 60-62].

In this regard, ancrod [63] from *Calloselasma rhodostoma* venom, whose current brand name is Viprinex™, was approved in 2005 in the fast track program of United States Food and Drug Administration (FDA) for investigating its use in patients suffering from acute ischemic stroke [64].

This program is currently undergoing phase III, where the patients received a one-time, 2-3 hour infusion of ancrod or placebo within six hours of the initial symptom onset of their ischemic stroke, and are then followed for three months to collect information on their functional status. Since then, many research articles about the use of ancrod in ischaemic stroke has been published [63,65-68].

Another thrombin-like enzyme that has been used clinically is Batroxobin (Defibrase®) from *Bothrops atrox* venom. In a randomized clinical trial using this toxin in association with aspirin indicated a reduced rate of restenosis in patients with diabetes undergoing angioplasty for lower-limb ischemia [69]. In another experiment, the combination of batroxobin and tranexamic acid in 80 adolescent patients undergoing scheduled idiopathic scoliosis surgery was able to markedly reduce blood loss and allogeneic blood transfusion [70]. Others trials involving batroxobin include deep vein thrombosis [71] treatment of hyperfibrinogenemia for secondary stroke prevention [72] and acute ischemic stroke [73].

Since SVSPs shortens the bleeding time and clotting time, by promoting coagulation locally at the site of bleeding, combination of enzymes is also employed for the prevention or treatment of hemorrhage such as might be encountered in surgeries. In this regard, hemocoagulase, a mixture of purified enzymes isolated from the venom of *Bothrops atrox* is another example in clinical trials [74,75]. It has two different enzymatic activities, one which promotes blood coagulation by converting prothrombin to thrombin (thromboplastin like enzymes) and the other that causes a direct transformation of fibrinogen to fibrin monomer.

Despite its use in clinical application, some SVSPs have also been explored as a diagnostic tool, mainly because they are not inhibited by heparin and therefore they can be used to test plasma samples containing this anticoagulant or to remove fibrinogen from samples containing heparin. In this context, Reptilase®, a thrombin-like serine protease isolated from the venom of *Bothrops atrox* is used to assess blood fibrinogen and fibrinogen degradation products [76,77]. It is useful to check whether a prolonged thrombin time is caused by the presence of heparin in the sample. However, the reptilase time is rarely performed in isolation and therefore, the results of this test should be considered together with other tests and in particular the thrombin time.

It is important to point out that the name "reptilase" was first described in 1958 [78] for an extract with the fibrinogen clotting activity from the venom of *Bothrops jararaca* and sometimes t his term has also been described as a synonymous of "batroxobin" from the venom of *Bothrops moojeni* and *Bothrops atrox*.

Protac®, a serine protease from *Agkistrodon contortrix* venom is another example that has found a broad application in diagnostic practice for the determination of disorders in the protein C (PC) pathway. Unlike thrombin-catalyzed PC activation reaction which requires thrombomodulin as a cofactor, Protac® directly converts the zymogen PC into the catalytically active form which can easily be determined by means of coagulation or chromogenic substrate techniques [79-81].

Due to the capability of a serine protease extracted from Russels´s viper (*Daboia ruselli*) venom (RVV-Vα) to activate Factor V, and since activated factor V is not stable and loses its activity within 20 hours at 37° C, RVV-V has been used to destabilize and selectively inactivate factor V in plasma. Therefore, it has been used to prepare a routine reagent for factor V determination. Studies have demonstrated the ability of the Prothrombinase-induced clotting time (PiCT) assay, which uses RVV-V among its components, to determine activities of both direct and indirect thrombin inhibitors in a linear manner over a wide concentration range [60-62].

While the original native snake venom compounds are usually unsuitable as therapeutics, interventions by medicinal chemists as well as scientists and clinicians in pharmaceutical R&D have made it possible to use snake toxins as therapeutics for multiple disorders based on the available structural and functional information. Therefore, snake venoms, with their cocktail of individual components, have great potential as therapeutic agents for human diseases [6].

2.4.1. Serine protease inhibitors

Most animal species synthesize a variety of protease inhibitors with different specificities, whose function is to prevent unwanted proteolysis. They generally act by unabling access of substrates to the proteases'active site through steric hindrance. Proteases are also involved in various disease states such as the destruction of the extracellular matrix of articular cartilage and bone in arthritic joints is thought to be mediated by excessive proteolitic activity [82]. Among the enzymes involved in extracellular matrix degradation, a few serine proteases (elastase, collagenase, cathepsin G) are able to solubilize fibrous proteins such as elastin and collagen [83,84].

Given the specific recognition by proteases of defined amino acid sequences, it may be possible to inhibit these enzymes when they are involved in pathological processes. Potent inhibitors have the potential to be developed as new therapeutic agents. In vertebrates for example serine protease inhibitors, have been studied for many years and they are known to be involved in phagocytosis, coagulation, complement activation, fibrinolysis, blood pressure regulation. Moreover, some of the protease inhibitors isolated from invertebrate sources are quite specific towards individual mammalian serine proteases. This also offers huge opportunities for medicine. Thus, the development of non-toxic protease inhibitors extracted from invertebrates for *in vivo* application may be quite important [82].

The last decade, drug discovery in leeches has opened the gate for new molecules to treat emphysema, coagulation, inflammation, dermatitis and cancer. Also other invertebrates, such as insects, harvest potential interesting molecules, such as serine protease inhibitors that can be exploited by the medical industry [85].

3. Conclusions

Snake venom serine proteases have several different functions and have found most use in medicine in blood coagulation system. These enzymes are used in several ways as tools in basic research helping to elucidate the relation of structure- function of coagulant proteins and their interactions with platelets or in experimental models of haemostatic alterations.

Some SVSPs have already been found to be a commercial use in coagulation diagnostic and some of them are used either to influence physiological homeostasis or as a form of supportive treatment in haemostatic disorders and micro vascular surgery promoting cicatrization.

Despite the high homology of serine proteases and even sharing the same target, small differences in their amino acids composition may lead significant binding intensity causing differences in their biological effects. Therefore, even isoforms of those molecules in the same organism must be explored. Many animals besides snakes also possess serine proteases that are used for attack or defense purposes, such as scorpions, bees, spiders and even the exotic platypuses which make 26 different kinds of serine proteases [86].

Therefore, the diversity of those toxins is extensive and demand many research to elucidate their function and potential clinical applications

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